SEROLOGICAL STUDIES OF THE ROOT-NODULE BACTERIA.

I. STRAINS OF RHIZOBIUM MELILOTI.

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Introduction.

Although a number of studies of the serological relationships of the root-nodule bacteria (*Rhizobium* spp.) have been undertaken (cit. Fred, Baldwin and McCoy, 1932), papers on this subject are comparatively rare in the more recent literature. Yet the earlier work gave some promising results both in confirming species differentiation and, more especially, in distinguishing strains of the one species, e.g., Stevens (1925) working with strains of *Rhizobium meliloti*.

Whilst several of the early workers included a variety of serological techniques, precipitation, complement fixation and agglutination, the latter was most used and is probably still the most convenient since the reaction is readily observed and a serum of adequately high titre can be obtained with little difficulty. Little has been done, however, in bringing to the study of *Rhizobium*, techniques which have given so much information in respect of pathogens, as, for example, the serological differentiation obtained within the Salmonella group by White and Kauffmann (Savage and White, 1925; White, 1926, and citations Topley and Wilson, 1936). Bushnell and Sarles (1939) point out the need for improved techniques and use three types of antigen: whole-cell, heated and saline extracted. Still, their results do not give a very clear picture of the antigenic constitution of the organisms with which they were working (from soybean and lupine), and they have made no differential flagellar and somatic analysis.

It seems likely that the serological reactions of the rhizobia will find more application as a means of differentiation between strains when we understand better the detail of the antigenic constitution of the cells, and attempt the correlation of this with other features of importance in the organisms' behaviour. Two simple points of technique merit further attention, viz.: (i) distinction between flagellar (*H*) and somatic (*O*) agglutination, and (ii) the application of serum absorption tests. So far as one is aware these refinements have not yet been reported with respect to *Rhizobium* although the keys to some of the tabulated results (Vogel and Zipfel, 1921; Wright, 1925) indicate that, at times, floccules characteristic of flagellar agglutination have been observed even though their significance has not been appreciated. The fact that readings were usually recorded after 24 hours would militate against distinction between *H* and *O* since, after that time, in the presence of both types, the agglutinated mass would be compacted.

This paper reports results when these techniques are applied to a number of strains of *Rh. meliloti* obtained from widespread areas and from various species of *Medicago*. Detailed attention has first been directed towards six organisms, for which antisera have been developed, in order to determine for these a minimal antigenic constitution—flagellar distinguished from somatic. These sera have then been used to study the serological relationships of 42 other strains.

METHODS.

Organisms used for the Development of Antisera.—These were selected to provide a variety of host species and to be representative of widely separated localities. Details are:

Collection No.	Host Plant.	Locality.
47	*Medicago sativa*	Bathurst, N.S.W.
74	*M. sativa*	Roseworthy, S. Aust.
27	*M. hispida* var. *denticulata*	Merrylands, N.S.W.
62	*M. hispida* var. *denticulata*	Roseworthy, S. Aust.
102	*M. arabica*	Dandenong, Vict.
66	*M. minima*	Tailem Bend, S. Aust.

All cultures were obtained from nodules during the latter half of 1939 by repeated single colony picking. They were further tested by plating on yeast mannitol agar containing congo red, growth on litmus milk and by streaking on potato slopes. In all cases these tests of purity were satisfactory and nodules were produced on inoculated lucerne plants.

Inoculating Antigen.—Growth from a yeast mannitol agar slope was rubbed off into about 5 ml. yeast mannitol solution (calcium carbonate omitted) and the suspension used to inoculate a slope of the same solid medium made up in a 4 oz. medicine bottle (flat). Slope cultures were incubated at 28°C., inclined so that a thin moist layer of inoculum covered the whole agar surface.

The suspension used for inoculating the animal contained about 500 million cells per ml. and was prepared by rubbing off the surface growth with sterile beads into sterile 0·85% saline. Younger (2-day) were mixed with older (5-day) growths to ensure a fair proportion of actively motile cells in the inoculum. Motility was checked by microscopic examination. The freshly prepared untreated suspension was inoculated intravenously into the ear of a rabbit in four increasing daily doses: 1 ml., 2 ml., 3 ml. and 3·5 ml.

Testing for "Normal" Antibodies.—A small sample of blood was collected from the ear of each animal before inoculation and the serum tested after separation against the organism used for that animal. In all cases the pre-inoculation serum failed to cause agglutination at a final dilution of 1/20.

Collection of Blood and Preparation of Serum.—Seven days after the completion of the course of injections, the presence of antibodies in the serum was checked using a small sample from the ear. Since these proved to be reasonably high, the principal yield of blood was obtained by bleeding out after a further three or four days. The serum was separated from the clot after a short incubation (1 hour at 37°C.) and standing over-night in a refrigerator. For storage, phenol was added to a final concentration of 0·5% and the serum kept in a refrigerator.

Dilution of Serum for Titrations.—The serum was diluted with 0·85% saline, usually starting at an initial dilution of 1/25 (= 1/50 after the addition of testing antigen in equal volume) and increasing four times at each dilution. This, whilst not giving a very accurate titre, covered the range quickly and was convenient for the purposes of this investigation where highly quantitative results would have no particular significance.

Agglutinating Antigen.—The organisms were grown on bottle slopes as in the case of the inoculating antigen; the shallow layer of liquid favoured the rapid development of an actively motile culture. As would be expected, cultures with few motile cells frequently gave poor, or no, *H* agglutination whereas the same strain taken earlier in development, when motile cells were abundant, provided a strong flocculent reaction. Two- to three-day cultures, grown at 28°C., were generally satisfactory although, in a few cases, 24- to 36-hour growths were markedly superior. Two types of agglutinating suspension were used:

(i) *Whole antigen*; mostly without any treatment although sometimes formolized to a concentration of 0·2% formalin. Whether untreated or formolized this antigen is capable of giving the two types of agglutination, viz., (*a*) typically flagellar, and (*b*) somatic.

(ii) *Heated antigen*; steamed for 30 minutes. The longer heating used by Bushnell and Sarles (1939) is not necessary to suppress *H*, and had a slight—though not marked—lowering effect on the *O* titre.

Because of the clumping tendency in *Rhizobium*, it was found advisable to give the suspension a light spinning (1,000 r.p.m. for 5 minutes) and to use the evenly turbid supernatant. In almost every case this treatment sufficed to give a stable suspension although some preparations gave a degree of "self-agglutination" in saline controls. This could yet be distinguished from typical agglutination, except perhaps causing one to be doubtful about the highest probably-agglutinating dilution. Greater stability in the one or two very badly "self-agglutinating" strains was obtained by using 0·5% instead of 0·85% saline for dilutions and preparation of suspension. In all cases saline-suspension controls were set up for comparison.

Effect of Centrifugation on Antigen Suspension.—In the course of absorption experiments, it was found that antigen reconstituted with saline from material concentrated by centrifuging and removing the supernatant (very slightly turbid) gave weak or no *H*-agglutination. The following case serves as an example:

Organism.	Treatment.	Dilutions of Serum 27.				
		1/25.	1/100.	1/400.	1/1,600.	1/6,400.
27	Untreated ..	H+	H+	H+	H+	H+
	Centrifuged ..	+	+	+	+	+
74	Untreated ..	H	H	H	H	H
	Centrifuged ..	(h)	(h)	−	−	−

H=full flocculent, (h)=very slightly flocculent, +=finely granular, − =no reaction.

A similar result was obtained in absorption when centrifuged material failed to remove completely antibodies to *H*. Centrifugation itself did not destroy the *H* antigen since a centrifuged preparation re-suspended in its own supernatant showed no loss of *H* agglutinability. Microscopic examination of the supernatant showed some motile organisms, but these seemed too few to account fully for the differences recorded above.

The Agglutination Test.—Equal parts of diluted serum and test antigen were mixed in Dreyer tubes and incubated at 53°C. in a water bath. Readings were taken after a shorter and longer period. Sometimes it was advantageous to include a one-hour or earlier observation for best recognition of *H* agglutination. Somatic agglutination was well developed after 2 hours, and, whilst a longer period served to confirm the results, it seldom added more than one further dilution step to the titre.

The two types of agglutination were sharply defined and perfectly normal. The flagellar was more rapid (usually evident within 15 minutes), markedly flocculent and, when without *O*, showed only partial clearing of the suspension. The somatic was very finely granular and gave typical complete clearing.

All readings were made by means of a reading box with oblique illumination and using a simple magnifying lens.

Absorption.—Preliminary trials indicated the need for a massive absorbing dose for satisfactory reduction of titre by the homologous organism. With the heavy dosage needed, the development of adequate supplies of antigen—at the same time young enough to contain a fair proportion of motile cells—presented a problem. Growth on slopes of moist medium proved superior to that in shallow liquid layers both for yield and motility. Attempts to concentrate absorbing antigen by centrifugation, removal of supernatant and making up to reduced volume with fresh saline showed that whilst absorption of *O* was thereby improved, it was impossible to remove the last of the *H*. For example, progressive exposure of serum 27 to doses of antigen concentrated in this way to 10×10^9 per ml. gave removal of *O* but a slight amount of *H* persisted at a dilution of 1/800 even after 50×10^9 cells had been applied per ml. of serum at 1/50.

It was found most practical to remove the *H* and *O* antibodies separately by means of the appropriate treatment. The *H* was best removed by using heavy (but not centrifuged) suspensions in the order of 3×10^9 cells per ml. of serum at a dilution of 1/50 or 1/100. Where this did not suffice for complete removal of *H*, the once-exposed supernatant was given a second treatment with the same suspension and the supernatant from this exposure tested with a few drops of concentrated testing antigen. The treatment for *H* did not usually suffice for complete exhaustion of *O* although the absorption effect could be seen in a lowering of titre and slowing down of reaction. The *O* antibodies were best removed by heavier doses (10×10^9 cells per ml. of serum at 1/50 or 1/100), using concentrated material prepared from sedimented cells.

The effect of three temperatures, 50°C., 37°C. and approximately 2°C., was compared. There was some indication that the highest temperature militated against complete absorption of *H* antibodies although the *O* were all removed after 2 hours. The refrigerator temperature was satisfactory provided exposure was prolonged overnight, and 37°C. was effective after 8 hours.

The Absorption Test.—The serum (usually at a dilution of 1/50) was mixed with the absorbing antigen and allowed to stand for the required time. The cells were then sedimented by centrifugation and the clear supernatant used to provide final dilutions—usually 1/100, 1/400 and 1/1,600. Control sera treated with (*a*) saline, and (*b*) homologous organism were used to check the testing antigen and conditions of absorption. In some cases it was useful to test the treated serum with the absorbing, as well as with the homologous organism, for removal of the particular antibodies with which the absorbing antigen was able to react.

RESULTS.

Cross-Agglutination Relationships of Six Strains studied in Detail.

Results of repeated tests of the six organisms used for the development of antisera are set out in Table 1. In each case the figure given takes account of at least two completely separate determinations carried out with a period of several months intervening. Agreement has been good both between duplicate tests and where the position of antigen and antiserum is reversed.

TABLE 1.
Cross-Agglutination Relationships of Six Strains.

Testing Antigen.	Sera. H-Reaction. 47	74	27	62	102	66	O-Reaction. 47	74	27	62	102	66
47	*3*	1	1	1	2	2	*4*	—	3	3	1	—
74	1	*4*	4	3	4	4	—	*3*	—	—	—	1
27	1	4	*4*	4	4	4	2	—	*4*	4	—	—
62	1	4	4	*4*	3	4	2	—	4	*4*	—	—
102	1	4	4	3	*4*	4	1	—	—	—	*4*	—
66*	—	4	4	3	4	*4*	—	—	—	—	—	*4*

* Antigen of 66, possibly due to its heavy gum production, gave some trouble with its *H* agglutinations.

Key: — = no reaction observed at 1/50 (final concentration of serum).
1 = positive reaction at 1/50 or 1/100; 2 = positive reaction at 1/200 or 1/400.
3 = positive reaction at 1/800 or 1/1,600; 4 = positive reaction at 1/3,200 or higher.

Flagellar Antigens.—74, 27, 62, 102 and 66 cross react to a high titre and 47 reacts with the others only to a low titre. It is not possible on the basis of this evidence to be more specific regarding *H.* agglutinogens; the present data do not justify the assumption that those of 74, 27, 62, 102 and 66 are identical. Absorption experiments are necessary to determine this point.

Somatic Antigens.—A consideration of these may be facilitated by a rearrangement of the *O* results and the use of symbols (Roman numerals) to represent a minimal antigenic constitution which can be deduced at this stage. These points are included in Table 2.

TABLE 2.
Somatic Cross Reactions.

	27	62	47	102	74	66	Minimal Antigenic Constitution.
27 *vs.*	4	4	2	—	—	—	I
62 *vs.*		4	2	—	—	—	I
47 *vs.*			4	1	—	—	I, III
102 *vs.*				4	—	—	III
74 *vs.*					3	1	II
66 *vs.*						4	II

Key: Numbers 1–4 and (—) as in Table 1.

Strains 27 and 62 have at least one common antigen (I) which is lacking in 102, 74 and 66. Strain 47 shares an antigen with 27 and 62 but must have at least one other not possessed by these but shared with 102. We may therefore assign to 47 the formula: I, III. Strains 74 and 66 share a common antigen not possessed by the others (II).

Low Titre Cross-Agglutinations.—It will have been noted that, in a number of cases, cross-agglutinations, both flagellar and somatic, together or separately, have been obtained only to a low titre (1/50 to 1/200). At the same time these agglutinations are quite definite and have all been carefully checked against saline controls of the suspension. There have been similar records by other workers, e.g., Vogel and Zipfel (1921) and Stevens (1925), but these workers have disregarded positive results at low titres or interpreted them as being due to "normal antibodies" contained in the sera of animals before inoculation. One feels that these results, because of the definite nature of the reaction observed and the uniform failure of the pre-inoculation sera to react at concentrations as high as 1/20, should be considered in drawing a picture of the antigenic constitution of the organism. It might be noted here that tests of eleven more sera from uninoculated rabbits were negative against the organisms being used for inoculation.

Absorption Tests of Antigenic Identity.

Absorption of Flagellar Antigens.—The kind of result obtained in numerous tests is illustrated in Table 3, which summarizes the absorptions between strain 27 and four other strains. The results are based on at least duplicate tests and where the absorption after one exposure was incomplete, a second exposure was given.

TABLE 3.

Absorptions of Flagellar Antibodies between 27 and 47, 74, 62, 102.

Serum.	Absorbed.	Tested.	Result.			Absorption.
			1/100.	1/400.	1/1,600.	
27	Saline	27	H+	H+	H+	
27	27	27	+	+–	–	Positive.
27	47	27	H+	H+	H+	Negative.
47	Saline.	47	H+	H+	H+	
47	47	47	(h)+	+	+–	
47	27	47	H+	H+	H+	Negative.
27	74	27	+	+	+	Positive.
74	Saline.	74	H+	H+	H	
74	74	74	+–	–	–	
74	27	74	+	+	–	Positive.
27	62	27	+	–	–	Positive.
62	Saline.	62	H+	H+	H+	
62	62	62	–	–	–	
62	27	62	+	–	–	Positive.
27	102	27	+	–	–	Positive.
102	Saline.	102	H+	H+	H+	
102	102	102	(h)+	(h)+	–	
102	27	102	(h)+	(h)+	–	Positive.

Key: H=full flocculent, (h)=very slightly flocculent, +=granular, – =no reaction, +– =slightly granular.

It is evident from these tests that 27, 62, 74 and 102 have qualitatively identical flagellar antigens whilst 47 has an antigen not possessed by the others and these have an antigen not possessed by 47. On this basis one can assign to 47 a minimal flagellar constitution of *Ab* and to the other four, *bC*.

Other cross absorption tests are confirmatory and results are summarized in Table 4. For simplicity any records of *O* agglutination are omitted.

TABLE 4.

Flagellar Absorptions between 47, 74, 62 and 102.

Test Dilutions.	Serum Absorbed and Testing Antigen.											
	47			74			62			102		
	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600
Absorbed by—												
Saline	H	H	H	H	H	h	H	H	H	H	H	H
47	h	—	—	H	H	h	H	H	H	H	H	H
74	H	H	h	—	—	—	—	—	—	—	—	—
62	H	H	h	—	—	—	—	—	—	h	—	—
102	H	H	—	—	—	—	—	—	—	h	—	—

Key: H = full flocculent, h = slight flocculent, — = no reaction.

Results with Strain 66.—Absorption tests with this strain have presented difficulties due to (i) gum production by the organism giving a very viscous serum after absorption, and (ii) a tendency for the testing antigen of strain 66 to give poor *H* agglutination, apparently due in part to loss of motility by the culture and in part to the gummy nature of the suspension. However, in a number of cases the difficulties were overcome and these satisfactory cases show identity with the *bC* strains:

Serum.	Absorbed.	Tested.	Result.		
			1/100.	1/400.	1/1,600.
74	66	74	(h)+	+	—
66	74	66	+	+	—
62	66	62	+	+	—
66	62	66	+	+	+
47	66	47	H+	H+	H+
66	47	66	H+	H+	H+

Key: As in Table 4.

Absorption of Somatic Antigens.—As noted above in connection with *O* cross-agglutinations, the six organisms provide a diverse collection of antigens. They may, however, be grouped thus:

(*a*) 27, 62 and 47, (*b*) 47 and 102, (*c*) 66 and 74.

The cross-absorptions within these groups are summarized in Table 5.

Interpretation of Results for Somatic Antigens.—Group (*a*): Cross-agglutination behaviour has already led to a formulation of I for 27 and 62 and I, III for 47. It would not be expected then that either 27 or 62 would absorb completely the serum of 47 and this was borne out by the results. Since the sera of 27 and 62 were not exhausted by 47, an additional antigen—not necessarily the same—will have to be postulated for these latter. Because 27 and 62 cross-absorb completely they can be written as I, IV.

Group (*b*): The addition of an extra antigen (V) to 102 will explain results.

Group (*c*): Since neither can absorb out the other each will require an extra antigen; 74 may then be written as II, VI and 66 as II, VII.

Agglutination Reactions of Other Cultures of Rhizobium meliloti *against Sera of Five Different Strains.*

In order to obtain a first picture of the antigenic relationships of a larger group of organisms their reactions were determined with the sera of strains 47, 74, 27, 102 and 66. Previous detailed investigation has shown that each of these has a different antigenic constitution. All tests have been carried out at least twice against four dilutions of each serum. Flagellar and somatic agglutination have been distinguished; in some cases heated and unheated suspensions were used, although it was easy to distinguish

TABLE 5.
Somatic Cross-absorption Tests.

Test Dilutions.	Serum Absorbed and Testing Antigen.																	
	27			62			47			102			66			74		
	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600	1/100	1/400	1/1,600
Absorbed by—																		
Saline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
27 } (a) ..	−	−	−	−	−	−	+	+	+									
62 } (a) ..	+*	−	−	−	−	−	+	+	+									
47 } (b) ..	+	+	+	+	+	+	±	−	−	+	+	+						
102 } (b) ..							+	+	±	−	−	−						
66 } (c) ..													±†	±†	−	+	+	−
74 } (c) ..													+	+	−	−	−	−

Key: + = granular agglutination, ± = slightly granular, − = no reaction.

* 27 abs. 62 and tested 62 gave a similar reaction at 1/100, an indication of incomplete absorption by 62 of its own antibody in serum 27.

† This reaction is very slow and slight compared with the others recorded for the same serum and its significance is doubtful.

each type even when present together and, except for confirmatory purposes, the use of heated antigen was discontinued.

In all but few cases the agreement between duplicates was good; in the case of disagreement one returned to cultures kept as reserve stocks for checking. In a few cases even young cultures failed to give an *H* reaction but, except for strain 101, when these were harvested at a time when active motility was observed good floccules were obtained with either one of the *H* groups.

For convenience the results are summarized in Table 6. The flagellar and somatic reactions are recorded separately and, in the case of the former, it is sufficient to indicate reactions with 47 and the remaining four, which have similar *H* antibodies, as a group exemplified by serum 27.

TABLE 6.
Agglutination Relationships of Various Strains of Rhizobium meliloti.

Collection No.	Host Plant.	Source.	Reactions with Test Sera. Flagellar.		Somatic.				
			47	27	47	74	27	102	66
27*	M.H.D.	Merrylands, N.S.W.	1	4	2	—	4	—	—
62*	M.H.D.	Roseworthy, S.A.	1	4	2	—	4	—	—
7†		Wisconsin (No. 101)	2	4	2	—	4	—	—
45	M.H.D.	Dandenong, Vic.	1	4	2	—	4	—	—
124‡	M.H.D.	Sydney, N.S.W. (J/M.d–U1)	1	4	2	—	4	—	—
24	M.S.	Leeton, N.S.W.	1	4	2	—	4	—	—
85	M.H.D.	Temora, N.S.W.	±	4	3	—	4	—	—
72	M.H.D.	Meningie, S.A.	1	4	3	—	4	—	—
125‡	M.H.D.	Vaucluse, N.S.W. (J/M.d-V) ..	1	4	3	—	4	—	—
31	M.A.	Manildra, N.S.W.	±	4	3	—	4	—	—
63	M.M.	Orange, N.S.W.	1	4	3	—	4	—	—

Key to species: M.A. = *Medicago arabica*, M.C. = *Medicago coerulea*, M.F. = *Medicago falcata*, M.G. = *Medicago gaetula*, M.H.D. = *Medicago hispida* var. *denticulata*, M.L. = *Medicago lupulina*, M.M. = *Medicago minima*, M.Mu. = *Medicago murex*, M.O. = *Medicago orbicularis*, M.S. = *Medicago sativa*, M.Sc. = *Medicago scutellata*, M.T. = *Medicago truncatula*.

Key to reactions: 4 = positive at 1/3,200 or higher, 3 = positive at 1/800, 2 = positive at 1/200, 1 = positive at 1/50, ± = inconsistent at 1/50, − = negative at 1/50.

* = Included from earlier section for comparison.

† = Supplied from another collection such as: 7 from Dept. of Agricultural Bacteriology, University of Wisconsin, their number being 101; 12 from N.S.W. Dept. of Agric. originally from U.S.A.

‡ = Supplied by Dr. H. L. Jensen, host plant and locality known; Jensen's number given thus (J/M.d–V) for No. 125.

TABLE 6.—*Continued.*

Agglutination Relationships of Various Strains of Rhizobium meliloti.—*Continued.*

Collection No.	Host Plant.	Source.	Reactions with Test Sera. Flagellar.		Somatic.				
			47	27	47	74	27	102	66
97	M.H.D.	Narrandera, N.S.W.	–	4	4	–	4	–	–
49	M.A.	Myrniong, Vic.	1	4	2	–	2	–	–
92	M.M.	Balranald, N.S.W.	1	4	3	–	2	–	–
75	M.H.D.	Bega, N.S.W.	1	4	3	–	2	–	–
128‡	M.L.	Canberra, A.C.T. (J/M.L–34)	–	3	4	–	2	–	–
79	M.M.	Euston, N.S.W.	1	4	2	–	–	–	–
74*	M.S.	Roseworthy, S.A.	1	4	–	3	–	–	1
134‡	M.S.	Canberra, A.C.T. (J/M–h–10)	1	4	–	3	–	–	±
102*	M.A.	Dandenong, Vic.	1	4	1	–	–	4	–
26	M.S.	Leeton, N.S.W.	1	4	1	–	–	4	1
126‡	M.A.	Canberra, A.C.T. (J/M.A–31)	–	4	–	–	–	3	–
89	M.H.D.	Hay, N.S.W.	1	4	–	–	–	–	1
133‡	M.Mu.	Canberra, A.C.T. (J/M.Mu–2)	–	4	4	–	4	–	1
112	M.S.	Wagga Wagga, N.S.W.	1	4	4	–	4	2	2
8†		Wisconsin (Nitragin P.)	1	4	1	2	2	2	3
12†		Dept. Agric., N.S.W. (U.S.A.)	1	4	3	2	2	2	3
14†		Dept. Agric., W.A.	1	4	2	2	2	2	3
15		Dept. Agric., N.S.W. (Wyong)	1	4	–	–	–	–	–
40	M.S.	Manildra, N.S.W.	1	4	±	–	–	–	–
122‡	M.S.	Lawes, Q. (J/M.S–C3)	–	3	–	–	–	–	–
51	M.S.	Blandford, N.S.W.	1	4	–	–	–	–	–
53	M.H.D.	Pinnaroo, S.A.	±	4	–	–	–	–	–
76	M.H.D.	Myrniong, Vic.	1	4	–	–	–	–	–
127‡	M.G.	Canberra, A.C.T. (J/M.G–1)	1	4	–	–	–	–	–
129‡	M.M.	Canberra, A.C.T. (J/M.M–164)	1	4	–	–	–	–	–
130‡	M.O.	Canberra, A.C.T. (J/M.O–35)	1	4	–	–	–	–	–
132‡	M.T.	Canberra, A.C.T. (J/M.Tr–90)	–	4	–	–	–	–	–
135‡	M.C.	Canberra, A.C.T. (J/M.C–1–3)	1	4	–	–	–	–	–
47*	M.S.	Bathurst, N.S.W.	3	1	4	–	3	1	–
131‡	M.Sc.	Canberra, A.C.T. (J/M.Sc–12)	3	1	2	–	3	–	–
25	M.S.	Leeton, N.S.W.	3	1	2	–	2	–	–
59	M.S.	Scone, N.S.W.	3	2	2	–	–	–	–
10†		Wisconsin (No. 109 G)	4	2	±	–	–	–	–
11†		Wisconsin (No. 107–1 G)	4	2	±	–	–	–	–
123‡	M.F.	Lawes, Q. (J/M.F–C1)	4	1	–	–	–	–	–
101	M.S.	Wingen, N.S.W.	–	–	–	–	–	–	–

Key to species of host plant: M.A.=*Medicago arabica*, M.C.=*Medicago coerulea*, M.F.=*Medicago falcata*, M.G.=*Medicago gaetula*, M.H.D.=*Medicago hispida* var. *denticulata*, M.L.=*Medicago lupulina*, M.M.=*Medicago minima*, M.Mu.=*Medicago murex*, M.O.=*Medicago orbicularis*, M.S.=*Medicago sativa*, M.Sc.=*Medicago scutellata*, M.T.=*Medicago truncatula*.

Key to reactions: 4=positive at 1/3,200 or higher, 3=positive at 1/800, 2=positive at 1/200, 1=positive at 1/50, ±=inconsistent at 1/50, –=negative at 1/50.

*=Included from earlier section for comparison.

†=Supplied from another collection such as: 7 from Dept. of Agricultural Bacteriology, University of Wisconsin, their number being 101; 12 from N.S.W. Dept. of Agric. originally from U.S.A.

‡=Supplied by Dr. H. L. Jensen, host plant and locality known; Jensen's number given thus (J/M.d–V) for No. 125.

On the basis of flagellar agglutination all but one of the forty-two strains examined fell into either of these two groups: (i) reacting weakly with serum 47 but strongly with 74, 27, 102 and 66 (35 out of 42 strains); (ii) reacting strongly with 47 but weakly with the sera of the other four (6 out of 42 strains). Strain 101, although successfully

re-tested for nodulation and re-isolated from these nodules, failed to give either an *H* or *O* with any of the five sera.

The somatic antigens give more division within this *H*-grouping. Within group (i) a proportionately large number (14/35) react with 47 and 27 (comparable with behaviour of 27 and 62), whilst two others react with either 66 (No. 133) or 102 and 66 (No. 112), in addition. One reacts strongly with serum 47, one with 74, two strongly with 102 and one, only slightly, with 66. Three organisms (8, 12 and 14) have an interestingly wide reactivity. The large group failing to react with any of the five sera for *O* indicates that even with the large number of antigens already postulated, the variety in somatic constitution is by no means exhausted. Within the group (ii), three out of six show little *O* reaction, 59 reacts with 47 only, and the remaining two strains show some similarity in reaction to strain 47 with which they are grouped in the table.

These results do not reveal any marked grouping according to locality; note, for example, the variety of areas supplying strains to the first and largest group. There is, however, some grouping possible on a host basis: three-quarters of those isolated from M.H.D. fall into the "27, 62" group and only one in eleven of those isolated from lucerne.

General Discussion.

The results presented in this paper emphasize the heterogeneous nature of strains of *Rhizobium meliloti*. The extent of such divergence is apparent from a separate consideration of the somatic and flagellar antigens and it will be interesting if it proves possible to connect one or other of these antigenic surfaces with characteristics of strain behaviour. For the present one has concentrated on the antigenic properties, but it is hoped to make collateral study of other characters a subject for further investigation.

It seems probable that the present division of the strains studied (Table 6) into groups showing some similarities in their behaviour with the five test sera, reveals only part of the serological constitution. Its full understanding requires a detailed investigation of the kind reported herein for six strains only. As a step in this direction, sera are now available for eleven organisms selected from those of Table 6; from these, more precise information should be forthcoming.

Summary.

A detailed serological analysis of six strains of *Rh. meliloti* requires the postulation of at least three flagellar and seven somatic antigens. Only two of the six strains appear to be identical and the antigens are shared as follows:

Strain.	H.	O.
47	*A b*	I, III
74	*b C*	II, VI
27, 62	*b C*	I, IV
102	*b C*	III, V
66	*b C*	II, VII

Tests of forty-two other strains against antisera of the five different strains above showed:

Wider grouping is possible on an *H* basis than on an *O*, the latter is more strain specific.

A fair proportion of the organisms possessed none of the *O* antigens postulated above.

A large proportion gave reactions very similar to strains 27 and 62 and this group contained about three-quarters of those isolated from *Medicago hispida* var. *denticulata* growing in widely scattered areas.

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